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Invited Review

miRNA in Wound Inflammation and Angiogenesis

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ABSTRACT

Chronic wounds represent a rising health and economic burden to our society. Emerging studies indicate that miRNAs play a key role in regulating several hubs that orchestrate the wound inflammation and angiogenesis processes. Of interest to wound inflammation are the regulatory loops where inflammatory mediators elicited following injury are regulated by miRNAs, as well as regulate miRNA expression. Adequate angiogenesis is a key determinant of success in ischemic wound repair. Hypoxia and cellular redox state are among the key factors that drive wound angiogenesis. We provided first evidence demonstrating that miRNAs regulate cellular redox environment via a NADPH oxidase-dependent mechanism in human microvascular endothelial cells (HMECs). We further demonstrated that hypoxia-sensitive miR-200b is involved in induction of angiogenesis by directly targeting Ets-1 in HMECs. These studies point toward a potential role of miRNA in wound angiogenesis. miRNA-based therapeutics represent one of the major commercial hot spots in today's biotechnology market space. Understanding the significance of miRs in wound inflammation and angiogenesis may help design therapeutic strategies for management of chronic nonhealing wounds.

Key words: miRNA, inflammation, angiogenesis, oxidants, redox

Abbreviations used: ARE, AU-rich elements; Bic, B-cell integration cluster; COX, cyclooxygenase; v-ets, Erythroblastosis virus E26 oncogene homolog 1 (Ets-1); HMEC, human microvascular endothelial cells; miRNAs, microRNAs; MCP-1/CCL2, CC chemokine macrophage chemoattractant protein; LT, leukotrienes; LX, lipoxins; PC, prostacyclin; PG, prostaglandins; ROS, reactive oxygen species; TX, thromboxanes; TLR, Toll-like receptors; TTP, tristetraprolin; VEGF, vascular endothelial growth factor.

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miRNA IN WOUND HEALING

Wound healing is a physiological response to injury that is conserved across tissue systems. Chronic wounds that fail to heal in an orderly manner represent a major health problem in the United States and costing in excess of US$25 billion annually [64]. For example, patients with a diabetic foot ulcer are seen by their outpatient healthcare provider about 14 times per year and are hospitalized about 1.5 times per year. The cost of care for these patients is estimated at $33,000 annually [36]. The discovery of miRs and their significance in biology represent a major breakthrough in molecular biology [5,12,15,22]. miR represents a key mechanism executing post-transcriptional gene silencing [47]. The human genome encodes 1048 microRNAs (miRNAs). As per estimates, 30–50% of the human protein-coding genes are regulated by miRs [27,31,58,74,87]. Key elements of tissue repair such as stem-cell biology, inflammation, hypoxia-response, and angiogenesis are all under the fine control of a network of wound-sensitive miRNAs [62,67]. Dysregulated response of the miR system to injury is likely to perturb the function of coding genes resulting in compromised wound healing. Therefore, it is necessary to develop a clear understanding of miR responses to wounding and their significance in specific aspects of healing.

Work in our laboratory has led to the maiden observation that cutaneous wound healing process involves changes in the expression of specific miRNA at various phases of healing [4,6,11,56,62,63,67,72,73]. We recently provided evidence on the significance of O_2-sensitive miRs in regulating cutaneous wound healing [6]. We also proposed the existence of regulatory loops where cytokines and other inflammatory mediators elicited following injury are regulated by miRNAs, which in turn regulate the expression of specific miRNA [56,62]. Adequate angiogenesis is a key determinant of success in ischemic wound repair. We demonstrated that miRNAs fine-tune the

cellular redox state as well as hypoxia-induced angiogenesis, key drivers of cell signaling in wound angiogenesis [73]. In this review article, we summarize the relevant literature that unveils the potential significance of miRNAs in the regulation of wound inflammation and angiogenesis.

miRNA IN WOUND INFLAMMATION

Wound-induced inflammatory response constitutes one of the earliest events that determine the fate and quality of healing (Figure 1) [14]. Cytokines, chemokines, and growth factors produced by infiltrating immune cells during early inflammatory phase set the stage for tissue repair. The inflammatory response in wound is tightly regulated by signals that either (i) initiate and maintain or (ii) resolve inflammation [51]. An imbalance between these signals may cause chronic inflammation derailing the healing cascade. Understanding the mechanisms that regulate the inflammatory response in wound repair will help design innovative strategies to address dysregulated inflammation as commonly noted in chronic ulcers. In the following section, we discuss the lines of evidence supporting that miRNAs regulate specific aspects of wound inflammation by targeting specific coding genes (Figure 2).

miRNAs and Inflammation-Related Target Genes

First, we discuss regulation of key cytokines and related factors by miRs (Table 1). TNF-α is known to be involved in tissue remodeling as well as mounting and sustenance of inflammation [57]. Depending on the concentration, length of exposure, and presence of other cytokines, the effect of TNF-α can be beneficial or deleterious for tissue repair. Anti-TNF-α therapy directed toward attenuating TNF-α signaling in wounds restores diabetic wound healing [19]. Suppression of inflammation is desired in such setting where inflammation is excessive and long-term. On the other hand, inability to mount an appropriate inflammatory process after wounding hurts wound healing too. We have recently observed that agonists of TNF-α production by wound macrophages can improve wound outcomes [52]. Post-transcriptional mechanisms impose a series of rate-limiting controls to modify the abundance of the TNF-α mRNA and the rate of its translation in response to inflammatory signals [76]. Such mechanisms consist of signaling networks converging on RNA-binding proteins as well as on miRNAs [76]. LPS-induced downregulation of miR-125b is instrumental in bolstering the production of TNF-α [81]. miR-125b has been shown to bind to the 3′-UTR of TNF-α inhibiting the translation of this cytokine [81]. In addition, the genes regulated by TNF-α, that is, E-selectin and ICAM-1, are direct targets of miR-31 and miR-17-3p, respectively [79].

Macrophage chemoattractant protein-1. The CC chemokine macrophage chemoattractant protein (MCP-1/CCL2) is a major chemoattractant for monocytes/macrophages. It also helps to recruit a subset of T cells and $CCR3^{+}$ mast cells [85]. The expression of MCP-1 was highly upregulated (∼70-fold) following wounding [55]. A putative consensus site for miR-124a binding in the 3′-UTR of MCP-1 mRNA has been identified. miR-124a specifically suppresses the reporter activity driven by the 3′-UTR of MCP-1 mRNA,

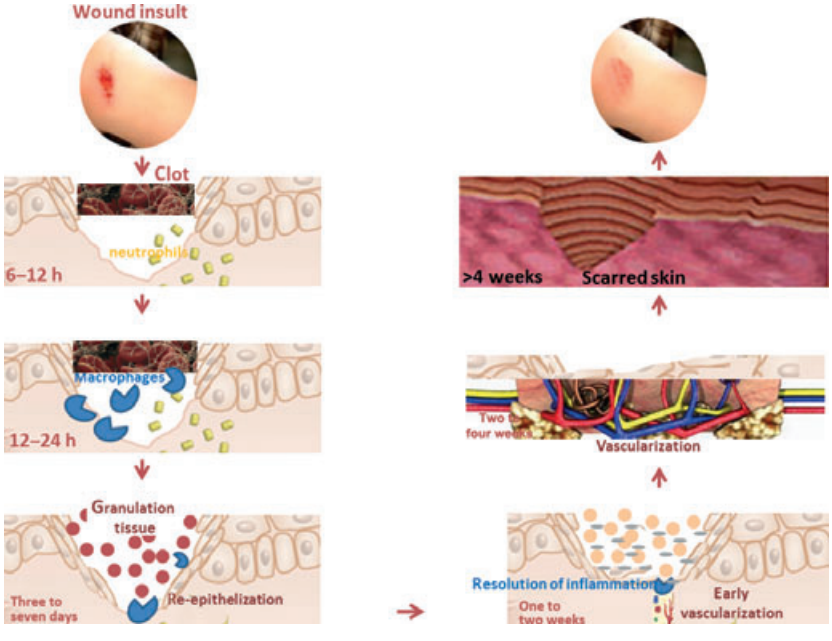

Figure 1. Phases of cutaneous wound healing. The process of wound healing for the ease of understanding is divided into specific functional phases, namely, hemostasis, inflammation, proliferation, and remodeling. All these phases of healing take place in an overlapping series of programmed events to promptly re-establish barrier function of the skin.

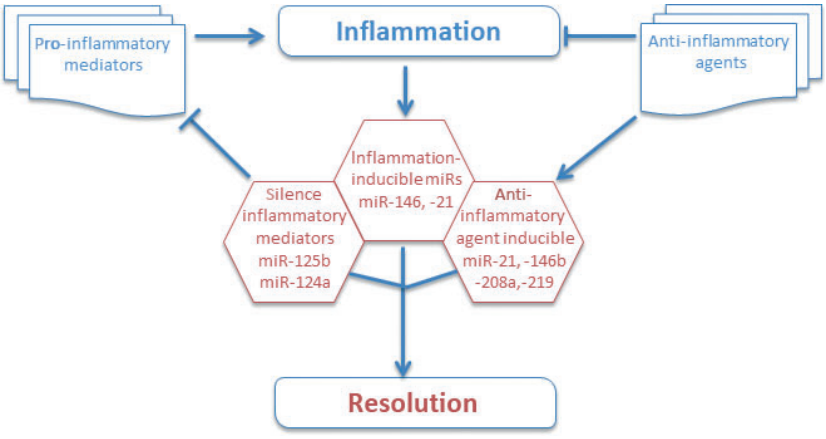

Figure 2. Potential role of miRNA in regulation of wound inflammation. The inflammation response to wound is tightly regulated by signals that either (i) initiate and maintain or (ii) resolve inflammation. An imbalance between these signals may cause chronic inflammation derailing the healing cascade. Of interest to wound inflammation are the regulatory loops where inflammatory mediators elicited following injury, are regulated by miRNAs as well as regulate miRNA expression.

Table 1. miRNA regulation of major proteins involved with wound inflammation

Cytokine	miRNA	References
TNF-α	miR-125b	[81]
TRAF6	miR-146a	[80]
IRAK	miR-146a	[80]
TGFβR1	miR-128a	[37]
IL-10	miR-466l	[34]
MCP-1	miR-124a	[41]

suggesting that miR-124a is directly implicated in the post-transcriptional silencing of MCP-1 [41].

Toll-like receptors. Inflammatory cells, including macrophages and neutrophils, recognize invading microbial pathogens primarily through TLRs [1]. Depending on the adaptor molecules recruited to the TLR intracellular domain after ligand engagement, TLR-activated signaling events are largely defined as myeloid differentiation primary response gene 88 (MyD88)-dependent or TIR-domain-containing adapter-inducing IFN-β (TRIF)-dependent [45]. MyD88-deficient mice exhibit severely impaired wound healing phenotype characterized by delayed granulation tissue formation and compromised blood vessel development independent of its role in host pathogen response [35]. miR-146a negatively regulates TLR signaling by targeting TRAF6 and IRAK-1, IRAK2 [22]. The miRNA-146 family (miR-146a/b) regulates TLR4 through a negative feedback loop mechanism [30]. IRAK1 and TRAF6 represent two prominent targets of miR-146a that enable negatively regulation of the release of IL-8 and RANTES [86]. In addition to TRAF6 and IRAK-1, IRAK2 has been identified as another target of miR-146a, which regulates IFN-γ production [23].

Lipid mediators. Lipid mediators such as eicosanoids consist of a family of biologically active metabolites, including PG, PC, TX, LT, and LX [20]. Free arachidonic acid is metabolized through the COX pathway, involving COX-1 and COX-2, along with terminal synthases, to generate PG, PC, and TX. Eicosanoids are well known to initiate, amplify, and perpetuate inflammation in both acute and chronic wounds [8]. The ω-3 poly unsaturated fatty acids, eicosapentaenoic (i.e., ω-3, C20:5), and docosahexaenoic acid (i.e., ω-3, C22:6) are transformed, in a manner equivalent to arachidonic acid metabolism, by COX-2 and lipoxygenase enzymes to generate novel classes of endogenous lipid autacoids with anti-inflammatory and protective function [20]. Induction of COX-2 represents one of the earliest responses following cutaneous injury [43]. miR-101a and miR-199a have been implicated in the inhibiting COX-2 expression in the murine uterus during embryo implantation [10].

Resolution of Inflammation

Cues and mechanisms that govern the resolution of inflammation play a key role in wound healing [51]. TGFβ1 and IL-10 represent major anti-inflammatory factors that direct the inflammation response following injury toward a successful resolution [51]. As a physiological response to wounding, TGFβ1 is released in large amounts from platelets. TGFβ1 serves as a chemoattractant for neutrophils, macrophages, and fibroblasts [85]. Signaling via active TGFβ involves recruitment of SMAD proteins [83]. SMAD proteins are now known to play a regulatory role in the processing of miRNA (miR biogenesis) into the nucleus [21]. Receptor-activated SMADs induce processing of a subset of miRNAs, particularly miR-21 [33]. Furthermore, miR-128a targets TGFβR1 protein expression by binding to the 3′-UTR region of this gene [37]. IL-10 is another major suppressor of the inflammatory response. It does so by downregulating the expression of proinflammatory genes

such as TNF-α [40]. Current evidence shows that insufficiency of IL-10 is a key factor underlying the exaggerated and sustained inflammatory response commonly noted in diabetic wounds [26]. In macrophages stimulated with TLR ligand, miR-466l can upregulate both mRNA and protein expression of IL-10 via competitive binding to the 3′-UTR that contains contain AU-rich elements (ARE). The RNA-binding protein tristetraprolin (TTP) mediates rapid degradation of IL-10 mRNA via binding to the ARE. Thus, binding of miR-466l to IL-10 ARE prevents TTP-mediated IL-10 mRNA degradation extending the half-life of IL-10 mRNA [34].

Lipid mediators, including LX, resolvins, protectin, and maresins, have been identified as key factors that are implicated in resolution of inflammation response [68]. These mediators are endogenously synthesized from essential fatty acids such as arachidonic acid during acute inflammation [68]. Recently, the anti-inflammatory lipid mediator Resolvin D1 has been shown to modify the expression of miRNAs such as miR-21, miR-146b, miR-208a, and miR-219 [49].

Expression and Regulation of miRNAs in Immune Cells

miR-21, miR-155, miR-424, and miR-17–92, and their transcriptional regulatory control are directly implicated in monocytic differentiation [59]. The relative levels of PU.1 and C/EBPα determine cell fate between monocyte and granulocyte as end products [48,60]. PU.1 activates the transcription of miR-424, stimulating monocyte differentiation through miR-424-dependent translational repression of the transcription factor NFIA. Ectopic expression of miR-424 in precursor cells enhances monocytic differentiation underscoring the significance of miR-424 in controlling the monocyte/macrophage differentiation program [50]. miR-223, preferentially expressed in myeloid cells [75], also plays an essential role in modulating the myeloid differentiation response [16]. Overexpression of miR-223 significantly increased the number of cells committed to the granulocyte-specific lineage in a granulocyte differentiation model. The loss of function study shows that miR-223 had the opposite effects on the differentiation process [16]. Furthermore, miR-223 is involved in an auto-regulatory feedback loop to control its own expression and enhance granulocytic differentiation [75]. These lines of evidence underscore the significance of miRNA in myeloid cell differentiation into active macrophages, a key driver of wound inflammation.

miRNA Regulated by the Inflammatory Response

miR-146, miR-155, and miR-21 have been of particular interest for research associated with inflammatory and immune responses. These miRNAs are induced by proinflammatory stimuli such as IL-1β, TNF-α, and TLRs [70]. The miR-146 family is composed of two members, miR-146a and miR-146b [86]. Promoter analysis studies recognized miR-146a as a NF-κB-dependent gene [80]. Exposure to proinflammatory cytokines such as TNF-α or IL-1β, or the ligands of TLR-2, -4, or -5 ligands (e.g., bacterial and fungal components) potently induces miR-146 expression in myeloid cells [66,80,81]. The ligands of TLR-3, -7, or -9 (e.g., single- or double-stranded RNA and CpG motifs) fail to induce miR-146 [86]. miR-155 represents a common target of a broad range of inflammatory mediators including TNF-α, LPS, polyriboinosinic:polyribocytidylic acid, and IFN-β [44]. miR-155 is encoded within an exon of the noncoding RNA known as bic (B-cell integration cluster). Bic null mice studies recognized miR-155 as a central regulator of lymphocyte differentiation [82]. Of note, IL-10 inhibits the LPS-inducible expression of miR-155 [38], while miR-21 or miR-146a remains unaffected. IL-10 inhibits the transcription of miR-155 from the BIC gene in a STAT3-dependent manner, thus allowing SHIP1 expression to recover and promote the conversion of PIP3 back to its inactive PIP2 state, switching off the proinflammatory response [38]. miR-21, initially described as ''oncomir,'' is known to be a common inflammation-inducible miR. The putative miR-21 promoter region contains three AP1 and one PU.1 binding sites [17]. Computational analyses predicted transcription repressor NFIB mRNA as a target for miR-21, and the miR-21 promoter itself contains a conserved binding site for the NFIB protein [17,25]. *In silico* analyses combined with experimental biology approaches have identified numerous target proteins whose expression is regulated by miR-21. PTEN represents major target of miR-21 [39]. Using laser-capture microdissection technique, we demonstrated that miR-21 signal was localized to cardiac fibroblasts of the infarcted region of the ischemia-reperfused heart. PTEN was identified as a direct target of miR-21 in cardiac fibroblasts [53]. Another target of miR-21 is proinflammatory PDCD4. A deceased level of PDDC4 is known to drive IL-10 production in response to LPS [71].

Inflammatory response such as TLR4 activation induces the expression of miR-125b. miR-125b, in turn, directly targets and silences TNF-α. This exemplifies a regulatory loop, where inflammatory response induces a specific miRNA, which in turn silences proinflammatory signals [81].

miRNA IN WOUND ANGIOGENESIS

Wound vascularization is controlled by all phases of wound healing—hemostasis, inflammation, tissue formation, as well as tissue remodeling (Figure 1). Early stages of wound vascularization include endothelial cell proliferation and migration followed by capillary formation where the sprouting of capillaries into the wound bed is critical to

support the regenerating tissue. Initial observations establishing the significance of miRs in guiding vascularization came from experimental studies involved in arresting miRNA biogenesis by Dicer knockdown in vascular cells and tissues to deplete available mature miR pools [29,69,73,78,88]. Dicer represents a key enzyme involved in miRNA biogenesis [24]. A key significance of miRNAs in the regulation of mammalian vascular biology was established from studies involved in blocking miRNA biogenesis to deplete the miRNA pools of vascular tissues and cell [29,78,89]. The dicer gene is significantly expressed throughout the embryonic tissues as early as day 11 and remains constant through day 17 [89]. Starting from embryonic day 11.5, virtually all homozygous dicer$^{ex1/2}$ (lacking the first two exons of *dicer* homozygous mutant mice) embryos were growth retarded and underdeveloped as compared with their wild type or heterozygous litter mates. The embryos that were still viable at this stage, however, had thin and sub-optimally developed blood vessels, providing evidence that miR is required for blood vessel development during embryogenesis [89]. Profound dysregulation of angiogenesis-related genes *in vitro* and *in vivo* was noticed after Dicer knock down [28,77]. Several aspects of angiogenesis, such as proliferation, migration, and morphogenesis of endothelial cell, are modified by specific miRNAs in an endothelial-specific manner (Figure 3) [62]. Endothelial miRs involved in angiogenesis, also referred to as angiomirs, include miR 17-5p, cluster 17-92, miR-15b, -16, -20, -21, -23a, -23b, -24, -27a, -29a,-30a, -30c, -31, -100, -103, -106, 125a and -b, -126, -181a, -191, -199a, -221, -222, -320, and let-7 family [9]. Angiomirs represent therapeutic targets, which may be manipulated to improve tissue vascularization outcomes.

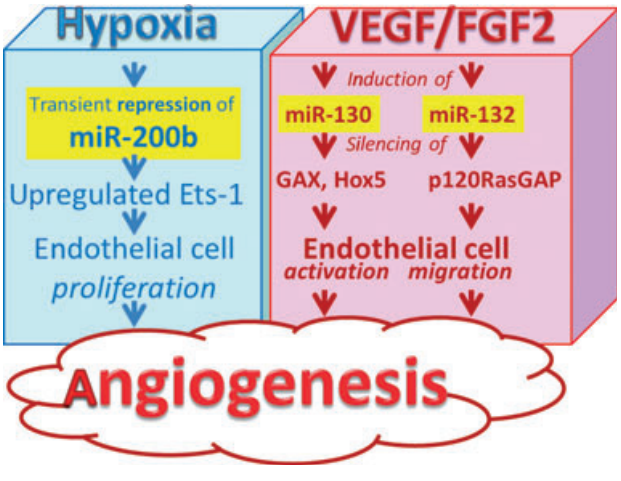

Figure 3. miRNAs in wound angiogenesis. Angiogenesis is a result of a cascade of events, which begins with the production and release of angiogenic factors like VEGF and FGF-2. Hypoxia also controls angiogenesis. Several aspects of angiogenesis, such as proliferation, migration, and morphogenesis of endothelial cell are modified by specific miRNAs. Endothelial miRs involved in angiogenesis are referred to as angiomirs.

miRNA Control of Redox and NADPH Oxidase

Oxidants generated during in inflammation may play a central role in supporting tissue vascularization [3]. Decomposition of endogenous H_2O_2 at the wound site by adenoviral catalase gene transfer impaired wound tissue vascularization [54]. Consistently, impairment in healing responses was noted in both NADPH oxidase-deficient mice and humans [54,61]. These and related studies point toward a central role of NADPH oxidase-derived reactive oxygen species (ROS) as signaling messengers in driving wound angiogenesis [65,66]. We examined whether redox control of angiogenesis is subject to regulation by miRNA. A Dicer knockdown approach was used to test the significance of miRNA in controlling redox state and angiogenic response of HMECs. Dicer knockdown resulted in lowering of mature miRNA pool and diminished the angiogenic response of HMECs as determined by cell migration and Matrigel tube formation. Such impairment of angiogenic response in the Matrigel was rescued by exogenous low micromolar H_2O_2. Dicer knockdown in HMECs showed lower inducible production of ROS when activated with phorbol ester, TNF-α, or vascular endothelial growth factor (VEGF). Limiting the production of ROS by antioxidant treatment or NADPH oxidase knockdown approaches impaired angiogenic responses. Lowered inducible ROS production following Dicer knockdown was associated with lower expression of p47phox protein in these cells. We identified that lowering of miRNA content by dicer knockdown resulted in the induced expression of the transcription factor HBP1, a suppressor transcription factor that negatively regulates p47phox expression. Knockdown of HBP1 restored the angiogenic response of miRNA-deficient HMECs [72,73]. This study provided the first evidence that cellular redox state, a key driver of cell signaling, is controlled by miRNAs. The results of this study lead to the hypothesis that miRNA may modify wound angiogenesis and therefore influence wound healing outcomes.

Hypoxia-Regulated miR Expression in Angiogenesis

The injured tissue often suffers from disrupted vasculature, leading to insufficient oxygen supply or hypoxia. Hypoxia is widely recognized as a cue that drives angiogenesis as part of an adaptive response to vascularize the oxygen-deficient host tissue. We noted that hypoxia-repressible miR-200b is involved induction of angiogenesis via directly targeting v-ets Erythroblastosis virus E26 oncogene homolog 1 (Ets-1) [11]. We reported that both hypoxia and HIF-1α stabilization inhibited miR-200b expression. In HMEC

cells, miR-200b-knockdown using miR-200b inhibitors exhibited elevated angiogenesis as evidenced by Matrigel® (BD Biosciences, Bedford, MA, USA) tube formation and increased cell migration. Conversely, delivery of the miR-200b mimic in HMECs inhibited the angiogenic response. Ets-1, a crucial angiogenesis-related transcription factor, served as a novel direct target of miR-200b. Certain Ets-1-associated genes, namely matrix metalloproteinase 1 and VEGF receptor 2, were silenced by miR-200b. Overexpression of Ets-1 rescued miR-200b-dependent impairment in angiogenic response and suppression of Ets-1-associated gene expression [11]. Taken together, the results demonstrate that transient downregulation of miR-200b helps jump-start wound angiogenesis.

Proangiogenic Stimuli

VEFG and FGF-2 represent two key stimuli that drive wound angiogenesis in a concerted manner. Immediately after injury, FGF-2 is released early, providing an early stimulus for endothelial cell proliferation. VEGF is produced as the FGF-2 levels decline. VEGF provides a more sustained stimulus for endothelial cell migration and differentiation into new capillary tubes [42]. VEGF-A has been shown to induce, in a time-dependent manner, the expression of miR-191, -155, -31, -17-5p, -18a, and miR-20a in HUVEC [78]. Both VEGF-A and basic FGF-2 increased the expression of miR-130a, a proangiogenic miRNA, which directly targets GAX and HOXA5 [13]. VEGF-A and bFGF signaling phosphorylate CREB causing rapid transcription of miR-132 [2]. miR-132 overexpression increased endothelial cell proliferation and *in vitro* networking by targeting p120Ras-GAP, a GTPase-activating protein [2]. miR-221 and miR-222 have been identified as modifying c-Kit expression as well as the angiogenic properties of the c-kit ligand Stem Cell Factor. The miR-221/2 and c-Kit interaction represents an integral component of a complex circuit that controls the ability of endothelial cells to form new capillaries [46]. Inhibition of c-kit results in reduced VEGF expression [32].

miRNA-BASED THERAPEUTICS

The role of miRNAs in a complex biological event such as inflammation and angiogenesis during wound healing is unfolding and remains to be fully understood. miRs lend themselves to clinical therapeutics [7,18] and are of extraordinary translational value [62]. Exploiting miRNAs for therapeutic purposes has great potential for two principal reasons: (i) a single miRNA can regulate multiple functionally convergent target genes, thus acting as an amplifier and (ii) miRNAs are relatively stable small molecules the tissue levels of which can be successfully manipulated by a growing number of technologies. Broadly, two major options are available: over-expression or silencing of the select miRNA. For the former, delivery of corrective synthetic miRNA in the form of (siRNA-like) dsRNA may be productive. For a disease phenotype caused by abnormal miRNA-dependent inhibition of a specific subset of mRNA, oligonucleotides complementary to either the mature miRNA or its precursors can be designed such that the miRNA will be functionally arrested and will not be able to bind the target mRNA subset. Successful design of such oligonucleotide should include considerations such as successful *in vivo* delivery, resistance to degradation in tissues, and specificity/high-binding affinity to the specific miRNA in question. This can be achieved by chemical modification of the nucleotides, especially the addition of chemical groups to the 2′-hydroxyl group [84]. The delivery of antagonists or mimics using viral and nonviral methods for gene therapy is of current interest and significant advances have been achieved through nanotechnology. Several companies are now developing miRNA-based therapeutics. Santaris Pharmaceuticals (San Diego, CA, USA) has launched a phase I clinical trial for the treatment of hepatitis C. The focus is on liver-specific miRNA-122, which is involved in hepatitis C replication and cholesterol metabolism. Regulus Therapeutics is developing therapies based on miR-122 inhibition to treat hepatitis C infection [69]. Regulus Therapeutics is also targeting miR-155 with anti-miRs to treat inflammatory diseases. miRNA-based therapies are lucrative as they provide fine tools enabling precise and temporally controlled manipulation of cell-specific miRNAs. Treatment of skin wounds has lower barriers because it lends itself to local delivery of miRNA mimics and antagonizing agents [62].

PERSPECTIVE

miRNAs are emerging as molecular switches that fine-tune signaling events controlling wound repair. Integral elements of tissue repair such as stem-cell biology, inflammation, hypoxia-response, and angiogenesis are all under the sophisticated control of a network of wound-sensitive miRNAs. Dysregulated response of the miR system to wounding will perturb the translation of coding genes resulting in compromised tissue repair. Therefore, it is necessary to develop a clear understanding of miR responses to wounding and their significance in specific aspects of the biology of healing. For therapeutic purposes, targeting miRNAs is lucrative because related technologies have substantially advanced demonstrating promising results in other disease systems.

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